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(71) Applicant (for all designated States except US): NOVOZYMES A/S [DK/DK]; Krogshøjvej 36, DK-2880 Bagsværd (DK).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BUDOLFSEN, Gitte [DK/DK]; Drosselvej 53M, DK-2000 Frederiksberg (DK). JENSEN, Morten, Tovborg [DK/DK]; Bringebakken 11, DK-3500 Værløse (DK). HELDT-HANSEN, Hans, Peter [DK/DK]; Vangeleddet 53, DK-2830 Virum (DK). STRINGER, Mary, Ann [US/DK]; Søborg Hovedgade 39C 3tv, DK-2860 Søborg (DK). LANGE, Lene [DK/DK]; Karensgade 5, DK-2500 Valby (DK).

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(54) Title: METHOD OF PREPARING A HEAT-TREATED PRODUCT

(57) Abstract: The formation of acrylamide during heat treatment in the production of a food product is reduced by treating the raw material with an enzyme before the heat treatment. The enzyme is capable of reacting on asparagine or glutamine (optionally substituted) as a substrate or is a laccase or a peroxidase.

METHOD OF PREPARING A HEAT-TREATED PRODUCT

FIELD OF THE INVENTION

The present invention relates to a method of preparing a heat-treated product with a low water content from raw material comprising carbohydrate, protein and water. It also relates 5 to an asparaginase for use in the method

BACKGROUND OF THE INVENTION

E. Tabeke et al. (*J. Agric. Food Chem.*, 2002, 50, 4998-5006) reported that acrylamide is formed during heating of starch-rich foods to high temperatures. The acrylamide formation has been ascribed to the Maillard reaction (D.S. Mottram et al., R.H. Stadtler et al., *Nature*, 10 419, 3 October 2002, 448-449).

WO 00/56762 discloses expressed sequence tags (EST) from *A. oryzae*.

Kim,K.-W.; Kamerud,J.Q.; Livingston,D.M.; Roon,R.J., (1988) Asparaginase II of *Saccharomyces cerevisiae*. Characterization of the ASP3 gene. *J. Biol. Chem.* 263:11948, discloses the peptide sequence of an extra-cellular asparaginase

15 SUMMARY OF THE INVENTION

According to the invention, the formation of acrylamide during heat treatment of raw material comprising carbohydrate, protein and water is reduced by treating the raw material with an enzyme before the heat treatment. Accordingly, the invention provides a method of preparing a heat-treated product, comprising the sequential steps of:

20 a) providing a raw material which comprises carbohydrate, protein and water
b) treating the raw material with an enzyme, and
c) heat treating to reach a final water content below 35 % by weight.

The enzyme is capable of reacting on asparagine or glutamine (optionally substituted) as a substrate or is a laccase or a peroxidase.

25 The invention also provides an asparaginase for use in the process and a polynucleotide encoding the asparaginase.

DETAILED DESCRIPTION OF THE INVENTION

Raw material and enzyme treatment

The raw material comprises carbohydrate, protein and water, typically in amounts of 30 10-90 % or 20-50 % carbohydrate of the total weight. The carbohydrate may consist mainly of starch, and it may include reducing sugars such as glucose, e.g. added as glucose syrup,

honey or dry dextrose. The protein may include free amino acids such as asparagine and glutamine (optionally substituted).

The raw material may include tubers, potatoes, grains, oats, barley, corn (maize), wheat, nuts, fruits, dried fruit, bananas, sesame, rye and/or rice.

5 The raw material may be in the form of a dough comprising finely divided ingredients (e.g. flour) with water. The enzyme treatment may be done by mixing (kneading) the enzyme into the dough and optionally holding to let the enzyme act. The enzyme may be added in the form of an aqueous solution, a powder, a granulate or agglomerated powder. The dough may be formed into desired shapes, e.g. by sheeting, cutting and/or extrusion.

10 The raw material may also be in the form of intact vegetable pieces, e.g. slices or other pieces of potato, fruit or bananas, whole nuts, whole grains etc. The enzyme treatment may comprise immersing the vegetable pieces in an aqueous enzyme solution and optionally applying vacuum infusion. The intact pieces may optionally be blanched by immersion in hot water, e.g. at 70-100°C, either before or after the enzyme treatment.

15 The raw material may be grain intended for malting, e.g. malting barley or wheat. The enzyme treatment of the grain may be done before, during or after the malting (germination).

The raw material before heat treatment typically has a water content of 10-90 % by weight and is typically weakly acidic, e.g. having a pH of 5-7.

Heat treatment

20 The process of the invention involves a heat treatment at high temperature to reach a final water content (moisture content) in the product below 35 % by weight, typically 1-20 %, 1-10 % or 2-5 %. During the heat treatment, the temperature at the surface of the product may reach 110-220°C, e.g. 110-170°C or 120-160°C.

25 The heat treatment may involve, frying, particularly deep frying in tri- and/or di-glycerides (animal or vegetable oil or fat, optionally hydrogenated), e.g. at temperatures of 150-180°C. The heat treatment may also involve baking in hot air, e.g. at 160-310°C or 200-250°C for 2-10 minutes, or hot-plate heating. Further, the heat treatment may involve kilning of green malt.

Heat-treated product

30 The process of the invention may be used to produce a heat-treated product with low water content from raw material containing carbohydrate and protein, typically starchy food products fried or baked at high temperatures. The heat-treated product may be consumed directly as an edible product or may be used as an ingredient for further processing to prepare an edible or potable product.

Examples of products to be consumed directly are potato products, potato chips (crisps), French fries, hash browns, roast potatoes, breakfast cereals, crisp bread, muesli, biscuits, crackers, snack products, tortilla chips, roasted nuts, rice crackers (Japanese "senbei"), wafers, waffles, hot cakes, and pancakes.

5 Malt (e.g. caramelized malt or so-called chocolate malt) is generally further processed by mashing and brewing to make beer.

Enzyme capable of reacting with asparagine or glutamine (optionally substituted) as a substrate

The enzyme may be capable of reacting with asparagine or glutamine which is optionally glycosylated or substituted with a peptide at the alpha-amino and/or the carboxyl position. The enzyme may be an asparaginase, a glutaminase, an L-amino acid oxidase, a glycosylasparaginase, a glycoamidase or a peptidoglutaminase.

The glutaminase (EC 3.5.1.2) may be derived from *Escherichia coli*. The L-amino acid oxidase (EC 1.4.3.2) capable of reacting with asparagine or glutamine (optionally glycosylated) as a substrate may be derived from *Trichoderma harzianum* (WO 94/25574). The glycosylasparaginase (EC 3.5.1.26, aspartylglucosaminidase, N4-(N-acetyl-beta-glucosaminyl)-L-asparagine amidase) may be derived from *Flavobacterium meningosepticum*. The glycoamidase (peptide N-glycosidase, EC 3.5.1.52) may be derived from *Flavobacterium meningosepticum*. The peptidoglutaminase may be peptidoglutaminase I or II (EC 3.5.1.43, EC 3.5.1.44).

20 The enzyme is used in an amount which is effective to reduce the amount of acrylamide in the final product. The amount may be in the range 0.1-100 mg enzyme protein per kg dry matter, particularly 1-10 mg/kg. Asparaginase may be added in an amount of 10-100 units per kg dry matter where one unit will liberate 1 micromole of ammonia from L-asparagine per min at pH 8.6 at 37 °C

25 **Asparaginase**

The asparaginase (EC 3.5.1.1) may be derived from *Saccharomyces cerevisiae*, *Candida utilis*, *Escherichia coli*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Fusarium graminearum*, or *Penicillium citrinum*. It may have the amino acid sequence shown in SEQ ID NO: 2 (optionally truncated to residues 27-378, 30-378, 75-378 or 80-378), 4, 6, 8, 10, 30 12 or 13 or a sequence which is at least 90 % (particularly at least 95 %) identical to one of these. It may be produced by use of the genetic information in SEQ ID NO: 1, 3, 5, 7, 9 or 11, e.g., as described in an example.

Whitehead Institute, MIT Center for Genome Research, Fungal Genome Initiative has published *A. nidulans* release 1 and *F. graminearum* release 1 on the Internet at <http://www-genome.wi.mit.edu/ftp/distribution/annotation/> under the *Aspergillus* Sequencing Project and

the *Fusarium graminearum* Sequencing Project. Preliminary sequence data for *Aspergillus fumigatus* was published on The Institute for Genomic Research website at <http://www-genome.wi.mit.edu/ftp/distribution/annotation/>.

The inventors inserted the gene encoding the asparaginase from *A. oryzae* into *E. coli* 5 and deposited the clone under the terms of the Budapest Treaty with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig. The deposit number was DSM 15960, deposited on 6 October 2003.

Alignment and identity

The enzyme and the nucleotide sequence of the invention may have homologies to 10 the disclosed sequences of at least 90 % or at least 95 %, e.g. at least 98 %.

For purposes of the present invention, alignments of sequences and calculation of identity scores were done using a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue 15 in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

20 Laccase or peroxidase

The laccase (EC 1.10.3.2) may be of plant or microbial origin, e.g. from bacteria or fungi (including filamentous fungi and yeasts). Examples include laccase from *Aspergillus*, *Neurospora*, e.g., *N. crassa*, *Podospora*, *Botrytis*, *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, e.g., *T. villosa* and *T. versicolor*, *Rhizoctonia*, e.g., *R. solani*, *Coprinus*, e.g., *C. cinereus*, *C. 25 comatus*, *C. friesii*, and *C. plicatilis*, *Psathyrella*, e.g., *P. condelleana*, *Panaeolus*, e.g., *P. papilionaceus*, *Myceliophthora*, e.g., *M. thermophila*, *Schytalidium*, e.g., *S. thermophilum*, *Polyporus*, e.g., *P. pinsitus*, *Phlebia*, e.g., *P. radita*, or *Coriolus*, e.g., *C. hirsutus*.

The peroxidase (EC 1.11.1.7) may be from plants (e.g. horseradish or soybean peroxidase) or microorganisms such as fungi or bacteria, e.g. *Coprinus*, in particular *Coprinus 30 cinereus* f. *microsporus* (IFO 8371), or *Coprinus macrorhizus*, *Pseudomonas*, e.g. *P. fluorescens* (NRRL B-11), *Streptoverticillium*, e.g. *S. verticillium* ssp. *verticillium* (IFO 13864), *Streptomyces*, e.g. *S. thermophilus* (CBS 278.66), *Streptomyces*, e.g. *S. viridospinus* (ATCC 39115), *S. badius* (ATCC 39117), *S. phaeochromogenes* (NRRL B-3559), *Pseudomonas*, e.g. *P. pyrrocinia* (ATCC 15958), *Fusarium*, e.g. *F. oxysporum* (DSM 2672) and *Bacillus*, e.g. *B. 35 stearothermophilus* (ATCC 12978).

Oxidoreductase capable of reacting with a reducing sugar as a substrate

The method of the invention may comprise treating the raw material with an oxidoreductase capable of reacting with a reducing sugar as a substrate. The oxidoreductase may be an oxidase or dehydrogenase capable of reacting with a reducing sugar as a substrate such as 5 glucose and maltose.

The oxidase may be a glucose oxidase, a pyranose oxidase, a hexose oxidase, a galactose oxidase (EC 1.1.3.9) or a carbohydrate oxidase which has a higher activity on maltose than on glucose. The glucose oxidase (EC 1.1.3.4) may be derived from *Aspergillus niger* e.g. having the amino acid sequence described in US 5094951. The hexose oxidase (EC 1.1.3.5) 10 may be derived from algal species such as *Iridophycus flaccidum*, *Chondrus crispus* and *Euthora cristata*. The pyranose oxidase may be derived from Basidiomycete fungi, *Peniophora gigantean*, *Aphyllophorales*, *Phanerochaete chrysosporium*, *Polyporus pinsitus*, *Bierkandera adusta* or *Phlebiopsis gigantean*. The carbohydrate oxidase which has a higher activity on maltose than on glucose may be derived from *Microdochium* or *Acremonium*, e.g. from *M. nivale* 15 (US 6165761), *A. strictum*, *A. fusidioides* or *A. potronii*.

The dehydrogenase may be glucose dehydrogenase (EC 1.1.1.47, EC 1.1.99.10), galactose dehydrogenase (EC 1.1.1.48), D-aldohexose dehydrogenase (EC 1.1.1.118, EC 1.1.1.119), cellobiose dehydrogenase (EC 1.1.5.1, e.g. from *Humicola insolens*), fructose dehydrogenase (EC 1.1.99.11, EC 1.1.1.124, EC 1.1.99.11), aldehyde dehydrogenase (EC 20 1.2.1.3, EC 1.2.1.4, EC 1.2.1.5). Another example is glucose-fructose oxidoreductase (EC 1.1.99.28).

The oxidoreductase is used in an amount which is effective to reduce the amount of acrylamide in the final product. For glucose oxidase, the amount may be in the range 50-20,000 (e.g. 100-10,000 or 1,000-5,000) GODU/kg dry matter in the raw material. One GODU 25 is the amount of enzyme which forms 1 µmol of hydrogen peroxide per minute at 30°C, pH 5.6 (acetate buffer) with glucose 16.2 g/l (90 mM) as substrate using 20 min. incubation time. For other enzymes, the dosage may be found similarly by analyzing with the appropriate substrate.

EXAMPLES**Media**

30 *DAP2C-1*

11g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

1g KH_2PO_4

2g Citric acid, monohydrate

30g maltodextrin

6g K₃PO₄·3H₂O

0.5g yeast extract

0.5ml trace metals solution

1ml Pluronic PE 6100 (BASF, Ludwigshafen, Germany)

5 Components are blended in one liter distilled water and portioned out to flasks, adding 250 mg CaCO₃ to each 150ml portion.

The medium is sterilized in an autoclave. After cooling the following is added to 1 liter of medium:

23 ml 50% w/v (NH₄)₂HPO₄, filter sterilized

10 33 ml 20% lactic acid, filter sterilized

Trace metals solution

6.8g ZnCl₂

2.5g CuSO₄·5H₂O

0.24g NiCl₂·6H₂O

15 13.9g FeSO₄·7H₂O

8.45g MnSO₄·H₂O

3g Citric acid, monohydrate

Components are blended in one liter distilled water.

Asparaginase activity assay

20 *Stock solutions*

50 mM Tris buffer, pH 8.6

189mM L-Asparagine solution

1.5 M Trichloroacetic Acid (TCA)

Nessler's reagent, Aldrich Stock No. 34,514-8 (Sigma-Aldrich, St. Louis, Mo. USA)

25 Asparaginase, Sigma Stock No. A4887 (Sigma-Aldrich, St. Louis, Mo. USA)

Assay

Enzyme reaction:

500 micro-l buffer

100 micro-l L-asparagine solution

30 350 micro-l water

are mixed and equilibrated to 37 °C.

100 micro-l of enzyme solution is added and the reactions are incubated at 37 °C for 30 minutes.

The reactions are stopped by placing on ice and adding 50 micro-l of 1.5M TCA.

The samples are mixed and centrifuged for 2 minutes at 20,000 g

Measurement of free ammonium:

50 micro-l of the enzyme reaction is mixed with 100 micro-l of water and 50 micro-l of Nessler's reagent. The reaction is mixed and absorbance at 436nm is measured after 1 minute.

Standard:

The asparaginase stock (Sigma A4887) is diluted 0.2, 0.5, 1, 1.5, 2, and 2.5 U/ml.

Example 1: Expression of an asparaginase from *Aspergillus oryzae* in *Aspergillus oryzae*

10 Libraries of cDNA of mRNA from *Aspergillus oryzae* were generated, sequenced and stored in a computer database as described in WO 00/56762.

The peptide sequence of asparaginase II from *Saccharomyces cerevisiae* (Kim,K.-W.; Kamerud,J.Q.; Livingston,D.M.; Roon,R.J., (1988) Asparaginase II of *Saccharomyces cerevisiae*. Characterization of the ASP3 gene. *J. Biol. Chem.* 263:11948), was compared to translations of the *Aspergillus oryzae* partial cDNA sequences using the TFASTXY program, version 3.2t07 (Pearson et al, *Genomics* (1997) 46:24-36). One translated *A. oryzae* sequence was identified as having 52% identity to yeast asparaginase II through a 165 amino acid overlap. The complete sequence of the cDNA insert of the corresponding clone (deposited as DSM 15960) was determined and is presented as SEQ ID NO: 1, and the peptide translated from this sequence, AoASP, is presented as SEQ ID NO: 2. This sequence was used to design primers for PCR amplification of the AoASP encoding-gene from DSM 15960, with appropriate restriction sites added to the primer ends to facilitate sub-cloning of the PCR product (primers AoASP7 and AoASP8, SEQ ID NOS: 14 and 15). PCR amplification was performed using Ex-tensor Hi-Fidelity PCR Master Mix (ABgene, Surrey, U.K.) following the manufacturer's instructions and using an annealing temperature of 55°C for the first 5 cycles and 65°C for an additional 30 cycles and an extension time of 1.5 minutes.

The PCR fragment was restricted with *BamHI* and *HindIII* and cloned into the *Aspergillus* expression vector pMStr57 using standard techniques. The expression vector pMStr57 contains the same elements as pCaHj483 (WO 98/00529), with minor modifications made to the *Aspergillus* NA2 promoter as described for the vector pMT2188 in WO 01/12794, and has sequences for selection and propagation in *E. coli*, and selection and expression in *Aspergillus*. Specifically, selection in *Aspergillus* is facilitated by the *amdS* gene of *Aspergillus nidulans*, which allows the use of acetamide as a sole nitrogen source. Expression in *Aspergillus* is mediated by a modified neutral amylase II (NA2) promoter from *Aspergillus niger* which is fused to the 5' leader sequence of the triose phosphate isomerase (tpi) encoding-gene from

Aspergillus nidulans, and the terminator from the amyloglucosidase-encoding gene from *Aspergillus niger*. The asparaginase-encoding gene of the resulting *Aspergillus* expression construct, pMStr90, was sequenced and the sequence agreed completely with that determined previously for the insert of DSM 15960

5 The *Aspergillus oryzae* strain BECh2 (WO 00/39322) was transformed with pMStr90 using standard techniques (Christensen, T. et al., (1988), Biotechnology 6, 1419-1422). Transformants were cultured in DAP2C-1 medium shaken at 200 RPM at 30°C and expression of AoASP was monitored by SDS-PAGE and by measuring enzyme activity.

Example 2: Purification of Asparaginase

10 Culture broth from the preceding example was centrifuged (20000 x g, 20 min) and the supernatants were carefully decanted from the precipitates. The combined supernatants were filtered through a Seitz EKS plate in order to remove the rest of the *Aspergillus* host cells. The EKS filtrate was transferred to 10 mM Tris/HCl, pH 8 on a G25 sephadex column and applied to a Q sepharose HP column equilibrated in the same buffer. After washing the Q sepharose 15 HP column extensively with the equilibration buffer, the asparaginase was eluted with a linear NaCl gradient (0 --> 0.5M) in the same buffer. Fractions from the column were analysed for asparaginase activity (using the pH 6.0 Universal buffer) and fractions with activity were pooled. Ammonium sulfate was added to the pool to 2.0M final concentration and the pool was applied to a Phenyl Toyopearl S column equilibrated in 20 mM succinic acid, 2.0M (NH₄)₂SO₄, 20 pH 6.0. After washing the Phenyl column extensively with the equilibration buffer, the enzyme was eluted with a linear (NH₄)₂SO₄ gradient (2.0 --> 0M) in the same buffer. Fractions from the column were again analysed for asparaginase activity and active fractions were further analysed by SDS-PAGE. Fractions, which was judged only to contain the asparaginase, were pooled as the purified preparation and was used for further characterization. The purified asparaginase was heterogeneously glycosylated judged from the coomassie stained SDS-PAGE 25 gel and in addition N-terminal sequencing of the preparation revealed that the preparation contained different asparaginase forms, as four different N-termini were found starting at amino acids A₂₇, S₃₀, G₇₅ and A₈₀ respectively of SEQ ID NO: 2. However, the N-terminal sequencing also indicated that the purified preparation was relatively pure as no other N-terminal sequences were found by the analysis.

Example 3: Properties of asparaginase

The purified asparaginase from the preceding example was used for characterization.

Asparaginase assay

A coupled enzyme assay was used. Asparaginase was incubated with asparagine 35 and the liberated ammonia was determined with an Ammonia kit from Boehringer Mannheim

(cat. no. 1 112 732) based on glutamate dehydrogenase and NADH oxidation to NAD⁺ (can be measured as a decrease in A₃₇₅). Hence the decrease in absorbance at 375 nm was taken as a measure of asparaginase activity.

Asparagine substrate :	10mg/ml L-asparagine (Sigma A-7094) was dissolved in Universal buffers and pH was adjusted to the indicated pH-values with HCl or NaOH.
Temperature :	controlled
Universal buffers :	100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CABS, 1 mM CaCl ₂ , 150 mM KCl, 0.01% Triton X-100 adjusted to pH-values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with HCl or NaOH.
Stop reagent :	500 mM TCA (Trichloroacetic acid).
Assay buffer :	1.0M KH ₂ PO ₄ /NaOH, pH 7.5.
Ammonia reagent A :	1 NADH tablet + 1.0 ml Bottle 1 (contain 2-oxoglutarate (second substrate) and buffer) + 2.0 ml Assay buffer.
Ammonia reagent B :	40 micro-l Bottle 3 (contain glutamate dehydrogenase) + 1460 micro-l Assay buffer.

5 450 micro-l asparagine substrate was placed on ice in an Eppendorf tube. 50 micro-l asparaginase sample (diluted in 0.01% Triton X-100) was added. The assay was initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which was set to the assay temperature. The tube was incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate (1400 rpm). The incubation was stopped by transferring the tube back to
 10 the ice bath and adding 500 micro-l Stop reagent. The tube was vortexed and centrifuged shortly in an icecold centrifuge to precipitate the proteins in the tube. The amount of ammonia liberated by the enzyme was measured by the following procedure: 20 micro-l supernatant was transferred to a microtiter plate, 200 micro-l Ammonia reagent A was added and A₃₇₅ was read (A₃₇₅(initial)). Then 50 micro-l Ammonia reagent B was added and after 10 minutes at room
 15 temperature the plate was read again (A₃₇₅(final)). A₃₇₅(initial) – A₃₇₅(final) was a measure of asparaginase activity. A buffer blind was included in the assay (instead of enzyme) and the decrease in A₃₇₅ in the buffer blind was subtracted from the enzyme samples.

pH-activity, pH-stability, and temperature-activity of asparaginase

The above asparaginase assay was used for obtaining the pH-activity profile, the pH-stability profile as well as the temperature-activity profile at pH 7.0. For the pH-stability profile the asparaginase was diluted 7x in the Universal buffers and incubated for 2 hours at 37°C.

After incubation the asparaginase samples were transferred to neutral pH, before assay for residual activity, by dilution in the pH 7 Universal buffer.

The results for the pH-activity profile at 37°C were as follows, relative to the residual activity at after 2 hours at pH 7.0 and 5°C :

pH	Asparaginase
2	0.00
3	0.01
4	0.10
5	0.53
6	0.95
7	1.00
8	0.66
9	0.22
10	0.08
11	0.00

5

The results for the pH-stability profile (residual activity after 2 hours at 37°C) were as follows:

pH	Asparaginase
2.0	0.00
3.0	0.00
4.0	1.06
5.0	1.08
6.0	1.09
7.0	1.09
8.0	0.92
9.0	0.00
10.0	0.00
11.0	0.00
12.0	0.00
	1.00

The results for the temperature activity profile (at pH 7.0) were as follows:

Temp (°C)	Asparaginase
15	0.24
25	0.39
37	0.60
50	0.81
60	1.00
70	0.18

Other characteristics

The relative molecular weight as determined by SDS-PAGE was seen as a broad band (a smear) at $M_r = 40\text{-}65$ kDa.

N-terminal sequencing showed four different terminals, corresponding to residues 27-5 37, 30-40, 75-85 and 80-91 of SEQ ID NO: 2, respectively.

Example 3: Cloning of asparaginase from *Penicillium citrinum*

Penicillium citrinum was grown in MEX-1 medium (Medium B in WO 98/38288) in flasks shaken at 150RPM at 26°C for 3 and 4 days. Mycelium was harvested, a cDNA library constructed, and cDNAs encoding secreted peptides were selected and sequenced by the 10 methods described in WO 03/044049. Comparison to known sequences by methods described in WO 03/044049 indicated that *Penicillium* sequence ZY132299 encoded an asparaginase. The complete sequence of the corresponding cDNA was determined and is presented as SEQ ID NO: 11, and the peptide translated from this sequence is presented as SEQ ID NO: 12.

15 Example 4: Effect of asparaginase on acrylamide content in potato chips

Asparaginase from *A. oryzae* having the amino acid sequence shown in SEQ ID NO: 2 was prepared and purified as in Examples 1-2 and added at various dosages to potato chips made from 40 g of water, 52.2 g of dehydrated potato flakes, 5.8 g of potato starch and 2 g of salt.

20 The flour and dry ingredients were mixed for 30 sec. The salt and enzyme were dissolved in the water, and the solution was adjusted to 30°C. The solution was added to the flour. The dough was further mixed for 15 min. The mixed dough was placed in a closed plastic bag and allowed to rest for 15 min at room temperature.

The dough was then initially compressed for 60 sec in a dough press.

25 The dough was sheeted and folded in a noodle roller machine until an approx. 5-10 mm dough is obtained. The dough was then rolled around a rolling pin and allowed to rest for

30 min in a plastic bag at room temperature. The dough was sheeted further to a final sheet thickness of approx 1.2 mm.

The sheet was cut into squares of approx 3 x 5 cm.

The sheets were placed in a frying basket, placed in an oil bath and fried for 45 sec at 5 180° C. The noodle basket was held at a 45° angle until the oil stopped dripping. The products were removed from the basket and left to cool on dry absorbent paper.

The potato chips were homogenized and analyzed for acrylamide. The results were as follows:

Asparaginase dosage U/kg potato dry matter	Acrylamide Micro-g per kg
0	5,200
100	4,600
500	3,100
1000	1,200
2000	150

10 The results demonstrate that the asparaginase treatment is effective to reduce the acrylamide content in potato chips, that the acrylamide reduction is clearly dosage dependent, and that the acrylamide content can be reduced to a very low level.

Example 5: Effect of various enzymes on acrylamide content in potato chips

Potato chips were made as follows with addition of enzyme systems which are capable of reacting on asparagine, as indicated below.

Recipe:

Tap water	40 g
Potato flakes dehydrated	52.2 g
Potato starch	5.8 g
Salt	2 g

Dough Procedure:

The potato flakes and potato starch are mixed for 30 sec in a mixer at speed 5. Salt and enzyme are dissolved in the water. The solution is adjusted to 30°C +/- 1°C. Stop mixer, 20 add all of the salt/enzyme solution to flour. The dough is further mixed for 15 min.

Place mixed dough in plastic bag, close bag and allow the dough to rest for 15 min at room temperature.

The dough is then initially compressed for 60 sec in a dough press.

The dough is sheeted and folded in a noodle roller machine until an approx. 5-10 mm dough is obtained. The dough is then rolled around a rolling pin and the dough is allowed to rest for 30 min in a plastic bag at room temperature. The dough is sheeted further to a final sheet thickness of approx 1.2 mm.

5 Cut the sheet into squares of approx 3 x 5 cm.

Sheets are placed in a frying basket, placed in the oil bath and fried for 60 sec at 180°C. Hold the noodle basket at a 45° angle and let the product drain until oil stops dripping. Remove the products from the basket and leave them to cool on dry absorbent paper.

The results from acrylamide analysis were as follows:

Enzyme	Enzyme dosage per kg of potato dry matter	Acrylamide Micro-g per kg
None (control)	0	4,100
Asparaginase from <i>Erwinia Chrysanthemi</i> A-2925	1000 U/kg	150
Glutaminase (product of Daiwa)	50 mg enzyme protein/kg	1,800
Amino acid oxidase from <i>Trichoderma harzianum</i> described in WO 9425574.	50 mg enzyme protein/kg	1,300
Laccase from <i>Myceliophthora thermophila</i> + peroxidase from <i>Coprinus</i>	5000 LAMU/kg + 75 mg enzyme protein/kg	2,000

10

The results demonstrate that all the tested enzyme systems are effective in reducing the acrylamide content of potato chips.

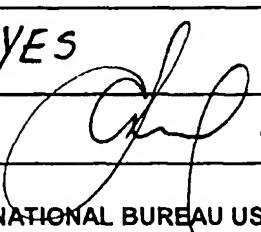
PCT

Original (for SUBMISSION) - printed on 10.10.2003 09:39:26 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis) Prepared using	PCT-EASY Version 2.92 (updated 01.07.2003)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	10347-WO

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on: page line	4 5-7
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	06 October 2003 (06.10.2003)
1-3-4	Accession Number	DSMZ 15960
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	YES 
0-4-1	Authorized officer	

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the International Bureau on:	
0-5-1	Authorized officer	

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE



INTERNATIONAL FORM

Novozymes A/S
Krogshojvej 36
DK-2880 Bagsvaerd

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITORY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR		II. IDENTIFICATION OF THE MICROORGANISM
Name: Novozymes A/S Krogshojvej 36 Address: DK-2880 Bagsvaerd		Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: DSM 15960
Date of the deposit or the transfer ¹ : 2003-10-06		
III. VIABILITY STATEMENT		
The viability of the microorganism identified under II above was tested on 2003-10-06 On that date, the said microorganism was		
<input checked="" type="checkbox"/> ² viable <input type="checkbox"/> ³ no longer viable		
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴		
V. INTERNATIONAL DEPOSITORY AUTHORITY		
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig		Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized official(s):
		Date: 2003-10-13

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

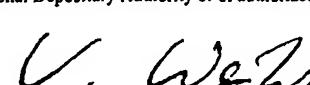
BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE



INTERNATIONAL FORM

Novozymes A/S
Krogshojvej 36
DK-2880 Bagsvaerd

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITORY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: NN049697	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: DSM 15960
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I above was accompanied by:</p> <p>(<input type="checkbox"/>) a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation</p> <p>(Mark with a cross where applicable).</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depository Authority accepts the microorganism identified under I. above, which was received by it on 2003-10-06 (Date of the original deposit)¹.</p>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
<p>The microorganism identified under I above was received by this International Depository Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on for conversion).</p> <p>(date of original deposit) (date of receipt of request)</p>	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized official(s):  Date: 2003-10-13

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

CLAIMS

1. A method of preparing a heat-treated product, comprising the sequential steps of:
 - a) providing a raw material which comprises carbohydrate, protein and water
 - b) treating the raw material with an enzyme capable of reacting on asparagine or glutamine (optionally substituted) as a substrate, a laccase or a peroxidase, and
 - c) heat treating to reach a final water content below 35 % by weight.
2. The method of the preceding claim wherein the enzyme capable of reacting on asparagine or glutamine (optionally substituted) as a substrate is an asparaginase, a glutaminase, an L-amino acid oxidase, a glycosylasparaginase, a glycoamidase (peptide N-glycosidase) or a peptidoglutaminase.
3. The method of the preceding claim wherein the asparaginase has an amino acid sequence which is at least 90 % identical to SEQ ID NO: 2 (optionally truncated to residues 27-378, 30-378, 75-378 or 80-378), 4, 6, 8, 10, 12 or 13.
4. The method of any preceding claim which further comprises treating the raw material with an oxidoreductase capable of reacting with a reducing sugar as a substrate.
5. The method of the preceding claim wherein the oxidoreductase capable of reacting with a reducing sugar as a substrate is a glucose oxidase, a pyranose oxidase, a hexose oxidase, a galactose oxidase (EC 1.1.3.9) or a carbohydrate oxidase which has a higher activity on maltose than on glucose.
- 20 6. The method of any preceding claim wherein the raw material is in the form of a dough and the enzyme treatment comprises mixing the enzyme into the dough and optionally holding.
7. The method of any preceding claim wherein the raw material comprises intact vegetable pieces and the enzyme treatment comprises immersing the potato pieces in an aqueous solution of the enzyme.
- 25 8. The method of any preceding claim wherein the raw material comprises a potato product.

9. A polypeptide having asparaginase activity and having an amino acid sequence which is at least 90 % identical with SEQ ID NO: 2 (optionally truncated to residues 27-378, 30-378, 75-378 or 80-378) or SEQ ID NO: 12.
10. A polynucleotide encoding the polypeptide of the preceding claim.
- 5 11. A polynucleotide which encodes an asparaginase and which comprises a nucleotide sequence which is at least 90 % identical to the coding sequences of SEQ ID NO: 1 or 11.

10347-WO-ST25
SEQUENCE LISTING

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Leu Gly Leu Leu Leu Ala Gln Gly Gly Lys Gly Thr Glu Glu Ile Arg		
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gcg gtg ttt ggg aag gtt gct gtt tgattcccgta ctgcccagggtt cttatgtatgt		1329
Ala Val Phe Gly Lys Val Ala Val		
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35 40 45		
Asn Thr Thr Leu Pro Asn Val Thr Ile Phe Ala Thr Gly Gly Thr Ile		
50 55 60		
Ala Gly Ser Ala Ala Ser Asn Thr Ala Thr Thr Gly Tyr Gln Ala Gly		
65 70 75 80		
Ala Leu Gly Ile Gln Thr Leu Ile Asp Ala Val Pro Glu Met Leu Ser		
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Val Ala Asn Ile Ala Gly Val Gln Ile Ser Asn Val Gly Ser Pro Asp		
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Val Thr Ser Thr Ile Leu Leu Glu Met Ala His Arg Leu Asn Lys Val		
115 120 125		
Val Cys Glu Asp Pro Ser Met Ala Gly Ala Val Val Thr His Gly Thr		
130 135 140		
Asp Thr Leu Glu Glu Thr Ala Phe Phe Leu Asp Ala Thr Val Asn Cys		
145 150 155 160		
Gly Lys Pro Ile Val Ile Val Gly Ala Met Arg Pro Ala Thr Phe Ile		
165 170 175		

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Ser Ala Asp Gly Pro Tyr Asn Leu Leu Gln Ala Val Thr Val Ala Ser
 180 185 190

Thr Lys Glu Ala Arg Asn Arg Gly Ala Met Val Val Met Asn Asp Arg
 195 200 205

Ile Ala Ser Ala Tyr Tyr Val Ser Lys Thr Asn Ala Asn Thr Met Asp
 210 215 220

Thr Phe Lys Ala Val Glu Met Gly Tyr Leu Gly Ala Ile Ile Ser Asn
 225 230 235 240

Thr Pro Phe Phe Tyr Tyr Pro Ala Val Gln Pro Ser Gly Lys Thr Thr
 245 250 255

Val Asp Val Ser Asn Val Thr Ser Ile Pro Arg Val Asp Ile Leu Tyr
 260 265 270

Ser Phe Gln Asp Met Thr Asn Asp Thr Leu Tyr Ser Ser Ile Glu Asn
 275 280 285

Gly Ala Lys Gly Val Val Ile Ala Gly Ser Gly Ala Gly Ser Val Asp
 290 295 300

Thr Ala Phe Ser Thr Ala Ile Asp Asp Ile Ser Asn Gln Gly Val
 305 310 315 320

Pro Ile Val Gln Ser Thr Arg Thr Gly Asn Gly Glu Val Pro Tyr Ser
 325 330 335

Ala Glu Gly Ile Ser Ser Gly Phe Leu Asn Pro Ala Lys Ser Arg
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atc atc aca ctc gcg gct atg ata gcc gtt ggg aat gcc tct ccg ttt Ile Ile Thr Leu Ala Ala Met Ile Ala Val Gly Asn Ala Ser Pro Phe	161
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gtc tac ccc cga gca acc agc cca aac agt aca tat gtc ttc acc aac Val Tyr Pro Arg Ala Thr Ser Pro Asn Ser Thr Tyr Val Phe Thr Asn	209
25 30 35	
tcg cat ggc ttg aac ttc acc cag atg aac acg acg ctc cct aat gtc Ser His Gly Leu Asn Phe Thr Gln Met Asn Thr Thr Leu Pro Asn Val	257
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acc atc ctc gca acc ggc ggt acc att gcc ggc tcc agc aac gac aac Thr Ile Leu Ala Thr Gly Thr Ile Ala Gly Ser Ser Asn Asp Asn	305
60 65 70	
acc gcc aca aca ggc tac acg gcc ggc gcg atc ggc atc cag cag ctc Thr Ala Thr Gly Tyr Thr Ala Gly Ala Ile Gly Ile Gln Gln Leu	353
75 80 85	
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90 95 100	
cag gtc gcc aat gtc ggc agc ccc gac gtg acg tct tcc ctt ctg ctc Gln Val Ala Asn Val Gly Ser Pro Asp Val Thr Ser Ser Leu Leu Leu	449
105 110 115	
cac atg gcc agg acc atc aac gag gtc gtc tgc gac gac ccc acc atg His Met Ala Arg Thr Ile Asn Glu Val Val Cys Asp Asp Pro Thr Met	497
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agc ggc gcc gtc atc acg cac ggc acc gac acg ctc gag gag acg gcc Ser Gly Ala Val Ile Thr His Gly Thr Asp Thr Leu Glu Glu Thr Ala	545
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ttc ttc ctc gac gct aca gtc aac tgc ggc aag ccc atc gtc gtc gtc Phe Phe Leu Asp Ala Thr Val Asn Cys Gly Lys Pro Ile Val Val Val	593
155 160 165	
ggc gcc atg cgg ccc gca acc gcc atc tcc gcc gac ggc ccg ttc aac Gly Ala Met Arg Pro Ala Thr Ala Ile Ser Ala Asp Gly Pro Phe Asn	641
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185 190 195	
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200 205 210 215	
tcc aag aca aac gcc aac acc atg gac acc ttc aag gcc gtc gag atg Ser Lys Thr Asn Ala Asn Thr Met Asp Thr Phe Lys Ala Val Glu Met	785
220 225 230	
ggc aac ctc ggc gcc atc atc tcc aac aag ccg tac ttc ttt tac ccg Gly Asn Leu Gly Ala Ile Ile Ser Asn Lys Pro Tyr Phe Phe Tyr Pro	833
235 240 245	
ccc gtc atg ccc acc ggt aag acc act ttc gac gtg cgc aac gtc gcc Pro Val Met Pro Thr Gly Lys Thr Thr Phe Asp Val Arg Asn Val Ala	881
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Ser Ile Pro Arg Val Asp Ile Leu Tyr Ser Tyr Gln Asp Met Gln Asn	
265 270 275	
gat acg ctc tac gac gcc gtc gac aac ggc gcg aaa ggc atc gtc gta a	978
Asp Thr Leu Tyr Asp Ala Val Asp Asn Gly Ala Lys Gly Ile Val Val	
280 285 290 295	
gtccagcccc tttctaaagc cctcacccgga tcaaccgctg aaattgaacc taatccagat	1038
cgcggctcc ggcgcag ga agc gtc tca agt ggc tac tac gat gcc atc	1087
Arg Ser Val Ser Ser Gly Tyr Tyr Asp Ala Ile	
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Asp Asp Ile Ala Ser Thr His Ser Leu Pro Val Val Leu Ser Thr Arg	
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Thr Gly Asn Gly Glu Val Ala Ile Thr Asp Ser Glu Thr Thr Ile Glu	
325 330 335	
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Ser Gly Phe Leu Asn Pro Gln Lys Ala Arg Ile Leu Leu Gly Leu Leu	
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Leu Ala Glu Asp Lys Gly Phe Lys Glu Ile Lys Glu Ala Phe Ala Lys	
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<211> 374

<212> PRT

<213> Aspergillus fumigatus

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Ser Thr Tyr Val Phe Thr Asn Ser His Gly Leu Asn Phe Thr Gln Met	
35 40 45	

Asn Thr Thr Leu Pro Asn Val Thr Ile Leu Ala Thr Gly Gly Thr Ile	
50 55 60	

Ala Gly Ser Ser Asn Asp Asn Thr Ala Thr Thr Gly Tyr Thr Ala Gly	
65 70 75 80	

Ala Ile Gly Ile Gln Gln Leu Met Asp Ala Val Pro Glu Met Leu Asp	
85 90 95	

Val Ala Asn Val Ala Gly Ile Gln Val Ala Asn Val Gly Ser Pro Asp	
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100	105	110
Val Thr Ser Ser Leu Leu Leu His Met Ala Arg Thr Ile Asn Glu Val		
115	120	125
Val Cys Asp Asp Pro Thr Met Ser Gly Ala Val Ile Thr His Gly Thr		
130	135	140
Asp Thr Leu Glu Glu Thr Ala Phe Phe Leu Asp Ala Thr Val Asn Cys		
145	150	155
Gly Lys Pro Ile Val Val Val Gly Ala Met Arg Pro Ala Thr Ala Ile		
165	170	175
Ser Ala Asp Gly Pro Phe Asn Leu Leu Gln Ala Val Thr Val Ala Ala		
180	185	190
His Pro Thr Ala Arg Asn Arg Gly Ala Leu Val Val Met Asn Asp Arg		
195	200	205
Ile Val Ser Ala Tyr Tyr Val Ser Lys Thr Asn Ala Asn Thr Met Asp		
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Thr Phe Lys Ala Val Glu Met Gly Asn Leu Gly Ala Ile Ile Ser Asn		
225	230	240
Lys Pro Tyr Phe Phe Tyr Pro Pro Val Met Pro Thr Gly Lys Thr Thr		
245	250	255
Phe Asp Val Arg Asn Val Ala Ser Ile Pro Arg Val Asp Ile Leu Tyr		
260	265	270
Ser Tyr Gln Asp Met Gln Asn Asp Thr Leu Tyr Asp Ala Val Asp Asn		
275	280	285
Gly Ala Lys Gly Ile Val Val Arg Ser Val Ser Ser Gly Tyr Tyr Asp		
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Thr Arg Thr Gly Asn Gly Glu Val Ala Ile Thr Asp Ser Glu Thr Thr		
325	330	335
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370

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 Met Ser Pro Ser
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 ttc cac tcc cta ctc gct atc gca acc ctt gca ggc tca gct gcc ctt 164
 Phe His Ser Leu Leu Ala Ile Ala Thr Leu Ala Gly Ser Ala Ala Leu
 5 10 15 20

gca tcc ccg atc ccg gag cca gaa aca ccg cag ctt atc ccc cgg gct 212
 Ala Ser Pro Ile Pro Glu Pro Glu Thr Pro Gln Leu Ile Pro Arg Ala
 25 30 35

gtt ggt gac ttt gag tgc ttc aac gct agt ctt ccc aac atc acc atc 260
 Val Gly Asp Phe Glu Cys Phe Asn Ala Ser Leu Pro Asn Ile Thr Ile
 40 45 50

ttc gcg act ggt ggt acc atc gct ggt tct gct ggt tct gcc gat cag 308
 Phe Ala Thr Gly Gly Thr Ile Ala Gly Ser Ala Gly Ser Ala Asp Gln
 55 60 65

act acg ggt tac cag gct ggt gca ttg ggt atc caa gcg ttg atc gac 356
 Thr Thr Gly Tyr Gln Ala Gly Ala Leu Gly Ile Gln Ala Leu Ile Asp
 70 75 80

gct gtc ccg caa ctc tgc aac gtc tcc aac gtc agg ggt gtg cag atc 404
 Ala Val Pro Gln Leu Cys Asn Val Ser Asn Val Arg Gly Val Gln Ile
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 Ala Asn Val Asp Ser Gly Asp Val Asn Ser Thr Ile Leu Thr Thr Leu
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gcg cat cgc atc cag act gat ctt gac aac cct cac atc caa ggt gtt 500
 Ala His Arg Ile Gln Thr Asp Leu Asp Asn Pro His Ile Gln Gly Val
 120 125 130

gtc gtc acc cat ggc aca gac act ctc gag gag tct tca ttt ttc ctc 548
 Val Val Thr His Gly Thr Asp Thr Leu Glu Glu Ser Ser Phe Phe Leu
 135 140 145

gat ctc act gtc caa agt gaa aag cct gtt gtt atg gtt gga tcc atg 596
 Asp Leu Thr Val Gln Ser Glu Lys Pro Val Val Met Val Gly Ser Met
 150 155 160

cgt cct gcc act gcc atc agc gct gat ggt ccc atc aac ctc ctg tct 644
 Arg Pro Ala Thr Ala Ile Ser Ala Asp Gly Pro Ile Asn Leu Leu Ser
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gct gtt cga ttg gca ggt agc aag agt gcc aag ggt cgc ggt aca atg 692
 Ala Val Arg Leu Ala Gly Ser Lys Ser Ala Lys Gly Arg Gly Thr Met
 185 190 195

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ggt gcc ttt gaa aac att cag ccc gtc ttc tgg tac cct gct agt cga Gly Ala Phe Glu Asn Ile Gln Pro Val Phe Trp Tyr Pro Ala Ser Arg 230 235 240	836
cca cta ggt cac cac tat ttc aac att agt gct agc tca cct aag aag Pro Leu Gly His His Tyr Phe Asn Ile Ser Ala Ser Ser Pro Lys Lys 245 250 255 260	884
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1260	

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<213> Fusarium graminearum
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20 25 30

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35 40 45

Asn Ile Thr Ile Phe Ala Thr Gly Gly Thr Ile Ala Gly Ser Ala Gly
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Ser Ala Asp Gln Thr Thr Gly Tyr Gln Ala Gly Ala Leu Gly Ile Gln
65 70 75 80

Ala Leu Ile Asp Ala Val Pro Gln Leu Cys Asn Val Ser Asn Val Arg
85 90 95

Gly Val Gln Ile Ala Asn Val Asp Ser Gly Asp Val Asn Ser Thr Ile
100 105 110

Leu Thr Thr Leu Ala His Arg Ile Gln Thr Asp Leu Asp Asn Pro His
115 120 125

Ile Gln Gly Val Val Val Thr His Gly Thr Asp Thr Leu Glu Glu Ser
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145 150 155 160

Val Gly Ser Met Arg Pro Ala Thr Ala Ile Ser Ala Asp Gly Pro Ile
165 170 175

Asn Leu Leu Ser Ala Val Arg Leu Ala Gly Ser Lys Ser Ala Lys Gly
180 185 190

Arg Gly Thr Met Ile Val Leu Asn Asp Lys Ile Ala Ser Ala Arg Tyr
195 200 205

Thr Val Lys Ser His Ala Asn Ala Val Gln Thr Phe Ile Ala Glu Asp
210 215 220

Gln Gly Tyr Leu Gly Ala Phe Glu Asn Ile Gln Pro Val Phe Trp Tyr
225 230 235 240

Pro Ala Ser Arg Pro Leu Gly His His Tyr Phe Asn Ile Ser Ala Ser
245 250 255

Ser Pro Lys Lys Ala Leu Pro Gln Val Asp Val Leu Tyr Gly His Gln
260 265 270

Glu Ala Asp Pro Glu Leu Phe Gln Ala Ala Val Asp Ser Gly Ala Gln
275 280 285

Gly Ile Val Leu Ala Gly Leu Gly Ala Gly Gly Trp Pro Asp Glu Ala
290 295 300

Ala Asp Glu Ile Lys Lys Val Leu Asn Glu Thr Asn Ile Pro Val Val
305 310 315 320

Val Ser Arg Arg Thr Ala Trp Gly Tyr Val Gly Glu Arg Pro Phe Gly
325 330 335

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 Met Met Pro Ser Val Arg Arg Phe His Gly Gln Thr
 1 5 10

atg gtc gcc gcc gct cct tct att tgc tca ggg cct gca gca tcg tcc 160
 Met Val Ala Ala Pro Ser Ile Cys Ser Gly Pro Ala Ala Ser Ser
 15 20 25

acc atc aag atg gct tca tcg tca gct tcg tgg acg act tat ctg tgg 208
 Thr Ile Lys Met Ala Ser Ser Ala Ser Trp Thr Thr Tyr Leu Trp
 30 35 40

cgg ctt atc cta gct gtg ctg gct cct tca acg gcc ctg ctg cct ttt 256
 Arg Leu Ile Leu Ala Val Leu Ala Pro Ser Thr Ala Leu Leu Pro Phe
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ggt gcg tgg gtt gtt tcg gtc tgg gga tct cct gtc ctc gac cta cac 304
 Gly Ala Trp Val Val Ser Val Trp Gly Ser Pro Val Leu Asp Leu His
 65 70 75

gtc caa cct cac ttc tcg gtt caa caa aaa gcg cca ata cag acg ggc 352
 Val Gln Pro His Phe Ser Val Gln Gln Lys Ala Pro Ile Gln Thr Gly
 80 85 90

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 Ile Pro Phe Glu Ile Ser Thr Thr Ser Gly Phe Asn Cys Phe Asn Pro
 95 100 105

aat ctt ccc aac gtc act att tat gcc acc gga ggt act att gct ggc 448
 Asn Leu Pro Asn Val Thr Ile Tyr Ala Thr Gly Gly Thr Ile Ala Gly
 110 115 120

tcc gca agc tcg gct gat cag acc acg gga tac cgg tca gct gcg tta 496
 Ser Ala Ser Ser Ala Asp Gln Thr Thr Gly Tyr Arg Ser Ala Ala Leu
 125 130 135 140

gga gtt gat tct ctc att gat gca gta ccc caa ttg tgc aat gta gcc 544
 Gly Val Asp Ser Leu Ile Asp Ala Val Pro Gln Leu Cys Asn Val Ala
 145 150 155

aat gtg aga ggt gtc cag ttt gcc aac acg gac agc ata gac atg agc 592

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160	165	170	
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Ser Ala Met Leu Arg Thr Leu Ala Lys Gln Ile Gln Asn Asp Leu Asp			
175	180	185	
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Ser Pro Phe Thr Gln Gly Ala Val Val Thr His Gly Thr Asp Thr Leu			
190	195	200	
gat gaa tct gcc ttc ttt ctg gat ctt act atc cag agc gac aag ccc			736
Asp Glu Ser Ala Phe Phe Leu Asp Leu Thr Ile Gln Ser Asp Lys Pro			
205	210	215	220
gtg gtc gtg aca ggc tca atg cgc ccg gca act gct atc agc gca gat			784
Val Val Val Thr Gly Ser Met Arg Pro Ala Thr Ala Ile Ser Ala Asp			
225	230	235	
gga cca atg aat ctt ttg tca tcg gtg aca ttg gca gca gca gcg agt			832
Gly Pro Met Asn Leu Leu Ser Ser Val Thr Leu Ala Ala Ala Ser			
240	245	250	
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Ala Arg Gly Arg Gly Val Met Ile Ala Met Asn Asp Arg Ile Gly Ser			
255	260	265	
gct cgt ttt acg acc aaa gtc aac gcc aac cat ttg gac gcc ttc caa			928
Ala Arg Phe Thr Thr Lys Val Asn Ala Asn His Leu Asp Ala Phe Gln			
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gcc cct gac agt ggc atg ctg gga aca ttc gtc aac gtt cag cca gtg			976
Ala Pro Asp Ser Gly Met Leu Gly Thr Phe Val Asn Val Gln Pro Val			
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ttt ttc tat ccg cca tca cga cct ctt ggc cac cgt cat ttt gat ctg			1024
Phe Phe Tyr Pro Pro Ser Arg Pro Leu Gly His Arg His Phe Asp Leu			
305	310	315	
cgg ccc atc acc aac aac ggc cgc cggtt gga cgc tct aca gcc ccc			1072
Arg Pro Ile Thr Asn Asn Gly Arg Arg Phe Gly Arg Ser Thr Ala Pro			
320	325	330	
gga gca gga tca tca gca cta ccc cag gtg gac gtg ctc tac gct tac			1120
Gly Ala Gly Ser Ser Ala Leu Pro Gln Val Asp Val Leu Tyr Ala Tyr			
335	340	345	
cag gag ctc agc gtg ggc atg ttc cag gcg gcc atc gac ctt gga gcg			1168
Gln Glu Leu Ser Val Gly Met Phe Gln Ala Ala Ile Asp Leu Gly Ala			
350	355	360	
cag ggc atc gtt cta gcg gga atg ggc gct gga ttc tgg acg tcc aaa			1216
Gln Gly Ile Val Leu Ala Gly Met Gly Ala Gly Phe Trp Thr Ser Lys			
365	370	375	380
ggt acc gag gag att cgg cgt atc gtc cac gag acc gat att ccc gtg			1264
Gly Thr Glu Glu Ile Arg Arg Ile Val His Glu Thr Asp Ile Pro Val			
385	390	395	
ata gtg agc cga aga ccg gaa ggc ggc ttc gtc gga cca tgt gag gca			1312
Ile Val Ser Arg Arg Pro Glu Gly Gly Phe Val Gly Pro Cys Glu Ala			
400	405	410	
gga atc ggc gcg ggc ttt ttg aat ccg caa aag gcg agg atc cag ctc			1360
Gly Ile Gly Ala Gly Phe Leu Asn Pro Gln Lys Ala Arg Ile Gln Leu			
415	420	425	
caa ctg gcc ctg gag acc aag atg gac aat gat gcc atc aaa gcc ctg			1408

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 <213> *Fusarium graminearum*

<400> 10

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Ala Ser Ser Ser Ala Ser Trp Thr Tyr Leu Trp Arg Leu Ile Leu
 35 40 45

Ala Val Leu Ala Pro Ser Thr Ala Leu Leu Pro Phe Gly Ala Trp Val
 50 55 60

Val Ser Val Trp Gly Ser Pro Val Leu Asp Leu His Val Gln Pro His
 65 70 75 80

Phe Ser Val Gln Gln Lys Ala Pro Ile Gln Thr Gly Ile Pro Phe Glu
 85 90 95

Ile Ser Thr Thr Ser Gly Phe Asn Cys Phe Asn Pro Asn Leu Pro Asn
 100 105 110

Val Thr Ile Tyr Ala Thr Gly Gly Thr Ile Ala Gly Ser Ala Ser Ser
 115 120 125

Ala Asp Gln Thr Thr Gly Tyr Arg Ser Ala Ala Leu Gly Val Asp Ser
 130 135 140

Leu Ile Asp Ala Val Pro Gln Leu Cys Asn Val Ala Asn Val Arg Gly
 145 150 155 160

Val Gln Phe Ala Asn Thr Asp Ser Ile Asp Met Ser Ser Ala Met Leu
 165 170 175

Arg Thr Leu Ala Lys Gln Ile Gln Asn Asp Leu Asp Ser Pro Phe Thr
 180 185 190

Gln Gly Ala Val Val Thr His Gly Thr Asp Thr Leu Asp Glu Ser Ala
 195 200 205

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Phe Phe Leu Asp Leu Thr Ile Gln Ser Asp Lys Pro Val Val Val Thr
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Gly Ser Met Arg Pro Ala Thr Ala Ile Ser Ala Asp Gly Pro Met Asn
 225 230 235 240

Leu Leu Ser Ser Val Thr Leu Ala Ala Ala Ala Ser Ala Arg Gly Arg
 245 250 255

Gly Val Met Ile Ala Met Asn Asp Arg Ile Gly Ser Ala Arg Phe Thr
 260 265 270

Thr Lys Val Asn Ala Asn His Leu Asp Ala Phe Gln Ala Pro Asp Ser
 275 280 285

Gly Met Leu Gly Thr Phe Val Asn Val Gln Pro Val Phe Phe Tyr Pro
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Pro Ser Arg Pro Leu Gly His Arg His Phe Asp Leu Arg Pro Ile Thr
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Asn Asn Gly Arg Arg Phe Gly Arg Ser Thr Ala Pro Gly Ala Gly Ser
 325 330 335

Ser Ala Leu Pro Gln Val Asp Val Leu Tyr Ala Tyr Gln Glu Leu Ser
 340 345 350

Val Gly Met Phe Gln Ala Ala Ile Asp Leu Gly Ala Gln Gly Ile Val
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Leu Ala Gly Met Gly Ala Gly Phe Trp Thr Ser Lys Gly Thr Glu Glu
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Gly Phe Leu Asn Pro Gln Lys Ala Arg Ile Gln Leu Gln Leu Ala Leu
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10347-WO-ST25

<213> Penicillium citrinum

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Asn Thr Ser Tyr Thr Asn Ser Asn Gly Leu Lys Phe Asn His Phe Asp		
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Gly Thr Ser Asp Asp Lys Thr Ala Thr Ala Gly Tyr Glu Ser Gly Ala		
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tct yca tct ctc tta ctg aac atg aca cat acc ctt caa aag acc gtt	387	
Ser Xaa Ser Leu Leu Asn Met Thr His Thr Leu Gln Lys Thr Val		
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Cys Asp Asp Pro Thr Ile Ser Gly Ala Val Ile Thr His Gly Thr Asp		
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Thr Leu Glu Glu Ser Ala Phe Phe Ile Asp Ala Thr Val Asn Cys Gly		
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Val Ser Ala Phe Phe Ala Thr Lys Thr Asn Ala Asn Thr Met Asp Thr		
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ttc aag gct tat gaa caa ggc agt ctt ggc atg att gtt tca aac aag	723	
Phe Lys Ala Tyr Glu Gln Gly Ser Leu Gly Met Ile Val Ser Asn Lys		
225 230 235		

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His Leu Asp Asp Val Asp Ala Ile Pro Arg Val Asp Ile Leu Tyr Ala	
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Tyr Glu Asp Met His Ser Asp Ser Leu His Ser Ala Ile Lys Asn Gly	
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Asp Phe Ser Asp Thr Ile Asp Glu Ile Ala Ser Lys His Gln Ile Pro	
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Asn Val Thr Leu Leu Ala Thr Gly Gly Thr Ile Ala Gly Thr Ser Asp
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Asn Arg Gly Ala Leu Val Val Leu Asn Asp Arg Ile Val Ser Ala Phe
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Phe Ala Thr Lys Thr Asn Ala Asn Thr Met Asp Thr Phe Lys Ala Tyr
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Glu Gln Gly Ser Leu Gly Met Ile Val Ser Asn Lys Pro Tyr Phe Tyr
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Tyr Pro Ala Val Glu Pro Asn Ala Lys His Val Val His Leu Asp Asp
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His Ser Asp Ser Leu His Ser Ala Ile Lys Asn Gly Ala Lys Gly Ile
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Thr Ile Asp Glu Ile Ala Ser Lys His Gln Ile Pro Ile Ile Leu Ser
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His Arg Thr Val Asn Gly Glu Val Pro Thr Ala Asp Ile Thr Gly Asp
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Asp Leu Ile Glu Ala Val Pro Ser Leu Ala Glu Lys Ala Asn Leu Asp
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Tyr Leu Gln Val Ser Asn Val Gly Ser Asn Ser Leu Asn Tyr Thr His
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Leu Ile Pro Leu Tyr His Gly Ile Ser Glu Ala Leu Ala Ser Asp Asp
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Tyr Ala Gly Ala Val Val Thr His Gly Thr Asp Thr Met Glu Glu Thr
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Ala Phe Phe Leu Asp Leu Thr Ile Asn Ser Glu Lys Pro Val Cys Ile
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Ala Gly Ala Met Arg Pro Ala Thr Ala Thr Ser Ala Asp Gly Pro Met
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Arg Gly Thr Met Ile Thr Leu Asn Asp Arg Ile Ala Ser Gly Phe Trp
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Gln Gly Tyr Leu Gly Tyr Phe Ser Asn Asp Asp Val Glu Phe Tyr Tyr
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Pro Pro Val Lys Pro Asn Gly Trp Gln Phe Phe Asp Ile Ser Asn Leu
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Thr Asp Pro Ser Glu Ile Pro Glu Val Ile Ile Leu Tyr Ser Tyr Gln
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Gly Leu Asn Pro Glu Leu Ile Val Lys Ala Val Lys Asp Leu Gly Ala
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Lys Gly Ile Val Leu Ala Gly Ser Gly Ala Gly Ser Trp Thr Ala Thr
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Gly Ser Ile Val Asn Glu Gln Leu Tyr Glu Glu Tyr Gly Ile Pro Ile
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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK 03/00684

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	A23L1/03	A21D8/04	A23L1/217	A23L1/105	C12N9/82
	C12N15/52				

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A23L A21D C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/28729 A (NOVONORDISK AS ; SI JOAN QI (DK)) 22 December 1994 (1994-12-22) claims 1,12,13,16,17 page 9, paragraph 1	1,4-6
X	WO 94/28728 A (NOVONORDISK AS ; SI JOAN QI (DK)) 22 December 1994 (1994-12-22) claims 1,5,11 page 8, paragraph 1	1,4-6
X	US 2002/004085 A1 (OLSEN HANS SEJR ET AL) 10 January 2002 (2002-01-10) the whole document	1,6-8

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

- *T* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

23 January 2004

Date of mailing of the International search report

09/02/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Vuillamy, V

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 03/00684

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/00029 A (NOVONORDISK AS ;WAGNER PETER (DK); NIELSEN PER MUNK (DK)) 8 January 1998 (1998-01-08) page 9, line 22 - line 31 page 6, line 29 - page 7, line 9 -----	1-5, 7, 9
X	WO 02/30207 A (BUDOLFSEN GITTE ;NOVOZYMES AS (DK); CHRISTIANSEN LUISE (DK)) 18 April 2002 (2002-04-18) claims; example 1 -----	1, 2, 6
X	US 6 039 982 A (SI JOAN QI ET AL) 21 March 2000 (2000-03-21) column 4, line 24 - line 39 column 6, paragraph 2 - paragraph '0003! claims -----	1, 2, 4-6
X	DATABASE WPI Section Ch, Week 199815 Derwent Publications Ltd., London, GB; Class D11, AN 1998-162469 XP002235162 & JP 10 028516 A (KAO CORP) 3 February 1998 (1998-02-03) abstract -----	1, 2, 4-6
X	PATENT ABSTRACTS OF JAPAN vol. 1997, no. 05, 30 May 1997 (1997-05-30) & JP 09 009862 A (CALPIS FOOD IND CO LTD:THE;AJINOMOTO CO INC), 14 January 1997 (1997-01-14) abstract -----	1
A	"Brief Communications" NATURE, vol. 419, 3 October 2002 (2002-10-03), pages 448-449, XP002235161 USA cited in the application the whole document -----	1
A	BIEKMAN E S A: "TOEPASSING VAN ENZYMEM BIJ DE VERWERKING VAN AARDAPPELEN TOT CONSUMPTIEPRODUKTEN" VOEDINGSMIDDELEN TECHNOLOGIE, NOORDERVLIET B.V. ZEIST, NL, vol. 22, no. 20, 12 October 1989 (1989-10-12), pages 51-53, XP000069625 ISSN: 0042-7934 the whole document -----	1, 4, 5, 7, 8
		-/-

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 03/00684

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/39828 A (DANISCO ; SOE JOERN BORCH (DK); PETERSEN LARS WEXOEE (US)) 23 May 2002 (2002-05-23) claims; example 11 -----	1
A	K.W. KIM: "Asparaginase II of Saccharomyces cerevisiae" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 24, 1988, pages 11948-11953, XP002266820 USA cited in the application the whole document -----	3

INTERNATIONAL SEARCH REPORT

Inter application No.
PCT/DK 03/00684

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/UK 03/00684

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
WO 9428729	A 22-12-1994	WO EP	9428729 A1 0701403 A1	22-12-1994 20-03-1996
WO 9428728	A 22-12-1994	AT DE DE WO DK EP ES GR PT US	188343 T 69422516 D1 69422516 T2 9428728 A1 702519 T3 0702519 A1 2142399 T3 3032941 T3 702519 T 6296883 B1	15-01-2000 10-02-2000 06-07-2000 22-12-1994 29-05-2000 27-03-1996 16-04-2000 31-07-2000 30-06-2000 02-10-2001
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JP 09009862	A 14-01-1997	NONE		
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